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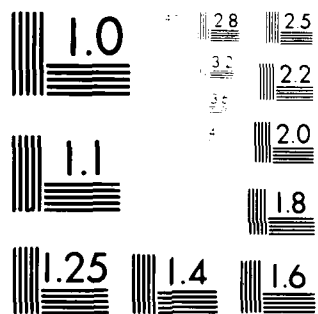
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The Pennsylvania State University
The Graduate School
Department of Comparative Medicine

The Effects of Acidification of Drinking Water
on Selected Biological Phenomena in Mice

A Thesis in
Laboratory Animal Medicine

by
James Edward Hall

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

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ABSTRACT

Acidification is used as a means of preventing bacterial growth in food and water, and is especially useful in preventing the transmission of bacterial disease through bottled drinking water. Few side effects have been reported using hydrochloric acid as the acidifying agent to a pH of 2.5. This study was done to determine if the consumption of water acidified with either hydrochloric or sulfuric acid to a pH of 2.0 or 2.5 produced significant effects in normal and immunosuppressed male random-bred mice. The phenomena evaluated included the following: water pH stability; weight; food and water consumption; state of hydration; stomach and small intestinal pH; stomach acid content; small intestine disaccharidase activity; liver microsomal enzyme activity; histopathologic tissue examination; and bacterial flora of the terminal ileum. Throughout the weekly test periods the pH of the drinking water remained stable. Weight gain was significantly reduced ($p < 0.05$) in the non-immunosuppressed mice receiving drinking water acidified to a pH of 2.0 with both acids. Weight gain of the immunosuppressed mice was also significantly reduced ($p < 0.05$). Water consumption was significantly reduced ($p < 0.05$) in male mice consuming pH 2.0 acidified water with both acids. These differences appeared to be due to the degree of acidification. The additional stress of immunosuppression increased the magnitude of these changes, and showed variation in effect between the different anions of the two acidifying agents. The anion group appeared to have its greatest effect on the bacterial flora of the terminal ileum, with a significant reduction ($p < 0.05$) in the number of bacterial species isolated from those mice receiving drinking water at pH 2.0 acidified

with sulfuric acid. This anion effect was also seen in the pH of the stomach contents from the immunosuppressed mice receiving water acidified with sulfuric acid at a pH of 2.0. No other significant changes were seen between the control and acid-treated groups in the parameters examined during this study. The changes observed suggest that the acidification of drinking water is not innocuous, and that it should be evaluated as an environmental variable whenever it is used.

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LIST OF ABBREVIATIONS

a.m.	Ante Meridiem
C	Centigrade
cm	Centimeter
F	Fahrenheit
g	A unit of force applied to a body at rest equal to the force exerted on it by gravity
ga	Gauge
gm	Gram
HCl	Hydrochloric acid
H ₂ SO ₄	Sulfuric acid
KCl	Potassium Chloride
M	Molar
μmol	Micromolar
mg	Milligram
ml	Milliliter
mM	Millimolar
min	Minute
N	Normal
NADP ⁺	Nicotinamide adenine dinucleotide phosphate, oxidized form
NaOH	Sodium Hydroxide
P	Probability
pH	The negative logarithm of the effective hydro- gen ion concentration
R	Rads
Tris-HCl	Tris (Hydroxymethyl) Aminomethane-Hydrochloride
%	Percent

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INTRODUCTION

The practice of acidifying drinking water with hydrochloric acid started during the 1950's from radiation studies utilizing mice. It was observed that within a few days of exposure to high levels of radiation, mice often became ill and died (15, 44, 52, 53). These deaths were most commonly associated with Pseudomonas aeruginosa, a ubiquitous opportunistic organism prevalent in soil, water, sewage and air. This organism was found to be highly pathogenic in immunosuppressed mice and has been associated with middle ear infection in non-immunosuppressed mice (15).

In 1957, Wensinck and his associates reported Pseudomonas aeruginosa to be a common contaminant of municipal water systems, which was seldom eliminated by chlorination or other commonly used methods of large-scale water treatment. They further postulated that the source of infection with Pseudomonas aeruginosa in irradiated mice was probably the drinking water (53). In addition to Pseudomonas aeruginosa contaminated drinking water from municipal water systems, carrier animals were shown to be capable of contaminating sterile water in the bottles from which they drank (11). Several methods for eliminating bacterial organisms including Pseudomonas aeruginosa from the drinking water were soon proposed and included: autoclaving of all equipment and water prior to use (11, 15, 53); thorough cleaning and hypochlorite sanitizing of equipment, (55); hyperchlorination of drinking water prior to use (2, 6, 7, 28, 41); the addition of antibiotics to water (17, 52); and the acidification of drinking water with hydrochloric acid at different pH levels below 3.0 (17, 29, 30,

37, 44). This problem focused attention on animal care practices resulting in an improvement in overall sanitation and a reduction in the incidence of Pseudomonas aeruginosa infection. It was noted, however, that hydrochloric acid treatment of the water appeared to be the most effective means of keeping the numbers of certain bacteria at a relatively low level in the water bottles for a period of up to seven days. This practice prevented the water in bottles from being a vector for transmission of bacterial disease (29, 30).

None of these studies involving consumption of water acidified with hydrochloric acid to a pH as low as 2.5 showed any apparent adverse effects on the animals. The parameters examined in these studies included: reproductive rate (2, 3), hexobarbital sleeping time (31), growth rate (27, 42), effect on dental enamel (43), the elimination of bacterial organisms from the feces (37), and histopathologic lesions (31). In contrast to these findings, measurable effects were found in animals consuming food acidified to pH levels below 3.5. The consequences of food acidification appear to be graded in magnitude with increasing levels of acid resulting in more extensive and profound effects. For example, dogs develop severe diarrhea following chronic acidification of their food with 0.1 N hydrochloric acid to a final pH of 3.5 (35). When the food was acidified below pH 2.5, in similar studies using rats and sheep, there was decreased food and increased water intake (19, 21, 45, 46). When the pH of the food was further reduced below 1.8 metabolic acidosis, reduced weight gain, bone resorption, and death occurred in rats and broiler chickens (1, 22, 24, 25, 33, 36).

Although acidification of food and drinking water is often done intentionally for specific experimental and disease control purposes in the laboratory environment, recent reports have indicated that widespread acid contamination from industrial pollution and naturally occurring sources has resulted in contamination of rivers, streams, and lakes with inorganic acids (20, 38, 39). Municipal water systems using these natural resources have reported pH levels between 3.0 and 4.0 associated with high chloride and sulfate ion levels. Without extensive water treatment to adjust the pH and ion content of the water, certain segments of the human and animal populations in the areas served by these water supplies may be exposed to chronic acid and ion loading. This situation would be similar to mice in a laboratory environment given acidified water to drink. Low pH of water has been shown to present a serious problem in those areas where water is drawn through pipes made of copper and lead, both potentially toxic heavy metals. Under such conditions leaching of these metals may occur with subsequent toxicosis in man and animals (39). It has been suggested that, in addition to the potentially harmful effects of low pH, excessive levels of chlorine may suppress the host defense mechanism in both man and animals, which may in turn lead to lowered host resistance to disease and neoplasia (10).

It is apparent from studies in both man and animals that subtle biochemical, metabolic and physiologic alterations which may result from the consumption of acidified drinking water have not been adequately studied. Environmental factors such as temperature, light, air quality, husbandry practices, and nutritional factors can alter the ability of the animal's body to metabolize exogenous compounds as well

as toxic waste products (12, 47, 49, 50, 51). Such seemingly innocuous factors can significantly affect experimental results.

Acidified water, if used routinely for all animals and not as a specific treatment for disease, is an integral but relatively unnoticed part of the environment of the laboratory animal. Any subtle effects that acidified water may cause then, can become important in the interpretation of experimental results. For this reason, it is important to study the effects of increased acid and ion load to the gastrointestinal tract of laboratory animals, and thus gain a better understanding of the magnitude and type of effects it may cause. It was the purpose of this study to determine if the degree of acidification or type of acidifying agent has the potential of producing significant changes in physiological or metabolic parameters including: body weight, food and water consumption, gastrointestinal pH, bacterial flora, and enzyme activities of the small intestinal mucosa and liver microsomes.

MATERIALS AND METHODS

Animals

A total of 132 male Crl:CD-1 mice¹ weighing 20-25 grams each were used in this study. The mice were housed in groups of six in stainless steel cages containing ground corn cob² as the bedding material. All animals were quarantined for 5 days prior to the initiation of the study. The animals were fed a standard laboratory diet³ ad libitum, and allowed free access to drinking water. The environment in the holding rooms in which they were housed was maintained at $72 \pm 2^{\circ}\text{F}$ ($21 \pm 1^{\circ}\text{C}$) with $50 \pm 20\%$ relative humidity and 18 ± 1 air changes per hour using 100% fresh air exchange. Light cycle in the holding rooms was automatically controlled to provide a 12-hour light (0700-1900): 12-hour dark (1900-0700) photoperiod without twilight.

Mice were assigned to treatment or control groups using a table of random numbers. Water was acidified to pH 2.0 or 2.5 by titration of deionized water with 1 N solutions of either hydrochloric or sulfuric acid using a pH meter⁴ and electrode⁵. Each experimental

¹Crl: COBS, CD-1(ICR)BR, Charles River Breeding Laboratories, Inc. Wilmington, MA 01887

²Sanicel, Paxton Processing Co., Inc., Paxton, IL 60957

³Wayne Lab-Blox 8604-00, Allied Mills Inc., Chicago, IL 60606

⁴Orion Research Model 701/Digital pH Meter, Orion Research, Inc., Cambridge, MA 02139

⁵Orion Model 91-02 Combination pH Electrode, Orion Research, Inc., Cambridge, MA 02139

group received only acid-treated deionized water using one acidifying agent at one of the two pH levels, while control groups received unaltered deionized water. All evaluations except liver microsomal enzymes were conducted on 5 groups of 6 mice, or a total of 30 mice in each of two phases (i.e., immunosuppressed and normal). The liver microsomal enzyme assays utilized 36 mice in each of the two phases.

Stability of Water pH

The stability of the acidified drinking water pH was determined by making biweekly measurements of the pH of the solutions used as the sole water source for each group. Fluctuations in pH were determined by calculating the difference between subsequent pH levels in each water bottle for each group of mice.

Drinking water was monitored for total bacterial numbers per milliliter using nutrient agar and the pour plate method (13).

Bacterial organisms present in the water were isolated from 0.1 ml samples added to thioglycollate enrichment medium⁶ followed by subculture on 5% sheep blood agar,⁶ Columbia colistin nalidixic acid agar,⁶ and MacConkey agar⁶ plates to separate individual organisms. Each organism was subsequently identified using standard microbiological laboratory techniques (3, 5).

Phase I - Non-Immunosuppressed Mice

Weight Change, Food and Water Consumption

Thirty male mice, six to a group, were weighed and had their food and water consumption measured three times per week for a period of

⁶BBL, Div. Becton, Dickinson & Co., Cockeysville, MD 21030

six weeks. Five groups of mice consisting of one control and four experimental groups were studied.

Water consumption was determined by measuring the weight of the solution consumed using a balance.⁷ The weight of solution consumed, to the nearest 0.1 gm, was the difference between subsequent measurements, and then dividing this amount by the number of days over which it was consumed. This difference in weights was then recorded as grams of water consumed per day. All animals in each cage were weighed collectively and recorded as total animal weight per cage. Individual animal weights were also recorded each time water consumption was measured. Total animal weight was used to calculate the water consumption per 100 gms of body weight per cage per day. Food consumption was determined by subtracting the amount of food remaining in the feeder from the amount given and the difference in weight divided by the number of days between measurements was recorded as grams of food consumed per day. The amount of food in grams consumed per day was then divided by the total animal weight per cage to yield daily food consumption per gram of body weight.

State of Hydration

Total body water was used as an indicator of the state of hydration. A modification of the technique described by Valtin (48) utilizing tritium labeled water⁸ was used during the fifth week of

⁷ Mettler Model P1200 Balance, Mettler Instrument Corp., Princeton, NJ

⁸ Water, Tritium Labeled, New England Nuclear, Pilot Chemicals Div., Boston, MA 02118

the food and water consumption portions of the study to measure the total body water content of the mice. All mice were individually injected intravenously in the dorsal tail vein with 0.1 ml of tritium labeled water diluted with sterile water⁹ to provide 3.4 microcuries of radioactivity per ml. Thirty minutes later blood samples were collected with microhematocrit capillary tubes¹⁰ from the left orbital sinus of each mouse. All samples were centrifuges in a capillary tube hematocrit laboratory centrifuge.¹¹ The serum portion of each tube was collected by scoring the capillary tube, breaking it, and blowing the serum into a test tube. A 0.1 ml serum sample from each animal was pipetted into empty scintillation vials¹² using a pipettor.¹³ Ten milliliters of scintillation counting solution¹⁴ was then added to each vial, capped, and thoroughly mixed. All vials were counted in a scintillation counter¹⁵ for 10 minutes, and the average counts per

⁹ Abbott Laboratories, North Chicago, IL 60064

¹⁰ Capilets Microhematocrit Tubes, Dade Div. American Hospital Supply Corp., Miami, FL 33152

¹¹ International Micro-capillary Centrifuge Model MB, International Equip. Co., Needham Heights, MA 02192

¹² Wheaton Liquid Scintillation Vials, Wheaton Scientific, Millville, NJ

¹³ Oxford Sampler^R Micropipetting System, Oxford Laboratories, Forest City, CA 94404

¹⁴ Aquasol^R, New England Nuclear, Pilot Chemicals Div., Boston, MA

¹⁵ Isocap/300 Liquid Scintillation System, Nuclear-Chicago, Des Plaines, IL

minute recorded. The amount of tritium labeled water administered was also counted by adding 0.1 ml of the injection solution to an empty vial plus 10 ml of counting solution, and then counted as above. Background radiation was measured by using only 10 ml of scintillation counting solution in a vial for counting. Calculation of body fluid compartment volume was made for each animal using the formula:

$$\text{Volume of compartment} = \frac{\text{Counts in 0.1 ml of injected solution}}{\text{Counts in 0.1 ml of serum after 30 min}}$$

Volumes were converted to milliliters, and divided by body weight for each animal to determine the ratio of body water to body weight.

Stomach and Small Intestinal pH, Stomach Acid Content, and Disaccharidase Activity

The remaining portions of this phase of the study, involving non-immunosuppressed male mice, were conducted after death of the animals. At the conclusion of the six-week food and water consumption study, the animals were fasted for 12 hours and then killed by cervical dislocation. Immediately after cervical dislocation, the abdominal cavities of all animals were opened aseptically with a ventral midline incision, coupled with transverse flank incisions to expose the abdominal contents. The ileocecal junction was then identified, and a two-centimeter section of the terminal ileum beginning at the ileocecal junction and extending proximally was aseptically removed and placed in a sterile container¹⁶ for subsequent bacteriological examination. The stomach was incised transversely at the junction of the glandular and non-glandular portions. The pH of the stomach contents was then measured in each stomach area

¹⁶Culturette^R Marion Scientific Corp., Rockford, IL 61101

using a pH meter and microelectrode¹⁷ allowing one minute for electrode equilibration.

Small intestine pH was measured in three locations: at a point two centimeters distal to the pylorus, at a point midway between the pylorus and the ileocecal valve, and at a point two centimeters proximal to the ileocecal junction. Following transverse incisions in these three locations the microelectrode was inserted 0.5 cm into the intestinal lumen and pH recorded allowing one minute for equilibration.

The stomach was then removed, and the contents flushed into a 20 ml beaker with 5.0 ml of deionized water and placed on ice for subsequent titration of acid content. Acid load of the stomach contents was measured by titration with 0.01N NaOH to a pH of 8.0. Results were expressed as milliequivalents of acid present in the stomach contents.

The small intestine was then excised from the incision 2 cm distal to the pylorus, to the incision previously made 2 cm proximal to the ileocecal junction. The intestinal contents were rinsed from each segment of the small intestine with 10 ml of maleate buffer (pH 6.0) at 4°C using a 10 ml syringe and 20 ga blunt tubing adapter. Maleate buffer was prepared by adding 4.0 ml of concentrated (17.5N) sodium hydroxide to 5.8 gm of maleic acid per 500 ml of deionized water, and adjusted to a pH of 6.0. The intestinal segments of each animal were then immersed in 10 ml of maleate buffer (pH 6.0), and placed in an ice bath for storage prior to mucosal stripping. Additional tissue samples were then collected for histologic examination.

¹⁷ MI-410 Micro-Combination pH Probe, Microelectronics, Inc., Grenier Industrial Village, Londonderry, NH 03053

Two to three hours later, the small intestinal segments were removed from the ice bath and opened throughout their full length to expose the mucosal surface, which was then examined for lesions. The mucosa was stripped off by scraping with a stainless steel spatula and placed in a glass homogenizer¹⁸ with 10 ml of ice cold maleate buffer (pH 6.0). The homogenization was carried out at 4°C to prevent enzyme degradation and consisted of six passes in a glass homogenizer with a glass pestle. Three aliquots of 3.0 ml each were separated for subsequent assay. The procedure of Dahlquist (9) as modified by White (54) was used to determine intestinal disaccharidase activity for sucrose and maltose.

Protein was determined by the Folin phenol method of Lowry et al. (26). The disaccharidase activities, expressed in terms of international units per gram of protein, were calculated and recorded for each group.

Pathologic Examination

A complete autopsy was done on each animal. Tissues taken for histopathological examination were fixed in 10% buffered formalin. Five micron paraffin sections were made from each of the tissues and stained with hematoxylin and eosin for examination by light microscopy (18).

Bacterial Flora of the Terminal Ileum

The aerobic and/or facultative bacterial flora from a segment of the ileum from each animal was qualitatively screened to examine possible changes resulting from acid water consumption. Initial isolation

¹⁸ Thomas Heavy Wall Tissue Grinder (10 ml), Arthur H. Thomas Co., Philadelphia, PA 19106

of organisms was obtained from swabs streaked on 5% sheep blood agar plates, MacConkey plates, and by swirling the swabs in tubes of thioglycollate medium without indicator. Each organism isolated was subsequently identified using standard bacteriological laboratory techniques (3,5,13).

Hepatic Microsomal Enzyme Activity

The hepatic microsomal enzyme activity phase of this study consisted of exposing one group of 18 mice to drinking water acidified with hydrochloric acid to a final pH of 2.0 for 10 days, and comparing microsomal enzyme activities with a group of 18 mice drinking only deionized water over the same period of time.

The liver microsomes were harvested for analysis between 7 and 8 a.m. in the morning following a 12-hour fast in accordance with the method of Brodie and Axelrod (4). Mice were killed by decapitation. The livers from three mice within each group were combined to provide sufficient material for testing. The pooled livers were weighed, and placed in two volumes of ice-cold 1.15% KCl solution buffered with 0.02 M Tris-HCl (pH 7.4). All subsequent tissue manipulations were carried out at 4°C to prevent enzyme degradation. Livers were homogenized by six passes in a glass homogenizer having a motor-driven teflon pestle. After homogenization the suspensions were transferred to polypropylene centrifuge tubes and spun at 9000 x g for 15 minutes in a refrigerated centrifuge.¹⁹ The fatty layer was aspirated from the top and the supernatant carefully transferred to high-speed polypropylene screw cap centrifuge tubes, leaving the pellet undisturbed in the

¹⁹ Sorvall Superspeed RC2-B, Ivan Sorvall Inc., Newton, CT 06470

bottom. The supernatant was then centrifuged at 78,000 x g in a refrigerated ultracentrifuge²⁰ for 60 minutes. The supernatant fraction from each high-speed ultracentrifugation was carefully decanted and discarded, leaving a layered pellet. The microsomal portion of each pellet was separated from the glycerol lower layer and resuspended in two volumes of buffered 1.15% KCl solution using a glass homogenizer. Final protein concentration after resuspension was in the range of 4 to 6 mg/ml as determined by the Folin phenol method of Lowry (26).

Microsomal Analytical Methods

The cytochrome P-450 content was determined by dithionate difference method of Omura and Sato (34), using a scanning dual beam spectrophotometer²¹. An extinction coefficient of $91 \text{ mM}^{-1} \text{ cm}^{-1}$ was utilized for the determination of cytochrome P-450 content immediately after microsome isolation.

Ethylmorphine N-demethylase activity was assayed in 3.0 ml of an incubation mixture composed of 15 μmol of ethylmorphine hydrochloride²², 1.31 μmol of NADP^{+23} , 25 μmol of magnesium chloride, 23.8 μmol of glucose-6-phosphate, and 0.99 units of glucose-6-phosphate dehydrogenase. Protein concentration was in the range from 2 to 3 mg per incubation

²⁰ Beckman Ultracentrifuge Model L5-65, Stanford Industrial Park, Palo Alto, CA

²¹ Aminco DW-2 UV/Vis Spectrophotometer, American Instrument Co., Div. Trivinal Labs., Inc., Silver Springs, MD 20910

²² Merck & Co., Rahay, NJ 07065

²³ Sigma Chemical Co., Phillipsburg, NJ 08865

vial. Formaldehyde produced during the demethylation process was measured according to the method of Nash (32) as corrected for formaldehyde formed in the absence of substrate by Gram (14). The mixtures were incubated in air at 37°C, using a shaking water bath (120 oscillations per minute) for 12 minutes.

Aniline hydroxylase activity was assayed in 3.0 ml of an incubation mixture composed of 65 μmol of aniline hydrochloride²⁴, 1.31 μmol of NADP⁺, 25 μmol of magnesium chloride, 32.8 μmol of glucose-6-phosphate, and 0.99 units of glucose-6-phosphate dehydrogenase. The concentration of protein was in the range of 4 to 6 mg/incubation vial. Aniline hydroxylase activity was monitored by assaying the amount of para-aminophenol formed as described by Chhabra *et al.* (8). The mixtures were incubated at 37°C for 22 minutes (120 oscillations per minute).

Microsomal enzyme activities were expressed as μmoles of para-aminophenol formed per gm microsomal protein per min for aniline hydroxylase, and μmoles of formaldehyde formed per gm of microsomal protein per min for ethylmorphine-N-demethylase. Cytochrome P-450, content was expressed as μmoles of cytochrome P-450 per gm of microsomal protein.

Phase II - Immunosuppressed Mice

In this phase of the study the mice were first exposed to 450 rads of gamma radiation from a cobalt 60 source²⁵ (40). The procedures and techniques previously described in Phase I were then repeated to

²⁴Eastman Kodak Co., Rochester, NY 14650

²⁵Gammacell 220, Atomic Energy of Canada Ltd.

determine the effect of acid water consumption on those parameters already described. These parameters included the following: weight gain, food and water consumption, hydrogen ion concentration of the stomach and small intestinal contents, gastric acid content, disaccharidase activity in the small intestinal mucosa, bacterial flora of the ileum, histologic examination of the tissues and liver microsomal enzyme activity. One group of six non-immunosuppressed mice was substituted for the six pH 2.5 sulfuric acid treated immunosuppressed mice.

Statistical Analysis

One factor analysis of variance were used to analyze the data from most portions of this study. Data from the non-immunosuppressed animals were analyzed using the five treatment groups (i.e. HCl 2.0, HCl 2.5, H_2SO_4 2.0, H_2SO_4 2.5, Control) as a between-groups factor. Data from the immunosuppressed animals were analyzed separately, since the groups were somewhat different in this phase of the study; (i.e. HCl 2.0, HCl 2.0, HCl 2.5, H_2SO_4 2.0, Control, plus a non-immunosuppressed control). Atain, the five treatment groups were treated as a between-groups factor.

Most dependent variables were measured individually for the six animals in each group in the immunosuppressed and non-immunosuppressed phases of this study, and analyses of these data were straightforward. Because it was necessary in both phases to house all animals from a given treatment group within a single cage it was impossible to obtain individual animal food and water consumption measurements. These data were therefore obtained by combining the immunosuppressed and non-immunosuppressed portions. The four treatment groups common to both

phases (i.e. HCl 2.0, HCl 2.5, H₂SO₄ 2.0, Control) were compared by one-way analysis of variance weekly for six weeks. Two data points were obtained for each treatment group during each week, one from the immunosuppressed and one from the non-immunosuppressed animals.

On all statistical tests a probability of 0.05 or less was considered statistically significant. Comparisons subsequent to significant overall F values were made using the Newman-Keuls procedure.

The liver microsomal enzyme activity was analyzed using the Students t test at a probability level of 0.05 to delineate significant differences between group means, since only two groups were tested.

The enumeration data from the bacterial flora isolations from the ileum was analyzed by the Chi-square test at a probability level of 0.05 to indicate significance.

RESULTS

Appearance and pH Stability of Drinking Water

Examination of the water bottles at weekly intervals, over the six weeks, showed varying amounts of suspended material, some of which could be identified as food particles, pieces of hair and pieces of bedding material. In addition, other material which could not be identified by gross examination was found suspended in the drinking water. This material was found in the drinking water from all groups.

In general, the water from animals receiving deionized drinking water was cloudy in appearance at the end of the seven-day observation periods. By contrast, the water acidified to pH 2.0 and 2.5 using either HCl or H_2SO_4 appeared relatively clear. Bacteriological culturing of this excess water after seven days' exposure to the mice revealed a variety of bacterial organisms (Table 1). Qualitative assessment of total bacteria per ml of water revealed substantially greater concentrations of bacteria in the deionized water as compared to acidified water.

The pH of the water remained relatively stable in all groups. Greater pH fluctuation was noted in the untreated deionized water than in any of the acid-treated water. The mean absolute fluctuation in deionized water pH was 0.20 ± 0.19 with a range of 0.15 ± 0.09 to 0.30 ± 0.17 . Mean pH of the deionized water was 6.23 ± 0.27 . Those receiving water acidified with either HCl or H_2SO_4 had mean absolute pH fluctuations of 0.035 ± 0.03 and a range of 0.0 ± 0.0 to 0.1 ± 0.07 . No statistically significant differences were observed when weekly pH fluctuations were compared within each treatment group.

TABLE 1
Numbers and Types of Bacteria in Water Bottles

Groups	Total Numbers of Organisms/ml of Water	Bacterial Genus Isolated
Control	$47.8 \pm 4.4 \times 10^6$ ^a	Pseudomonas ^b Escherichia Proteus Staphylococcus Bacillus
HCl 2.0	None	None
HCl 2.5	$45.5 \pm 1.2 \times 10^3$	Pseudomonas Bacillus
H ₂ SO ₄ 2.0	None	None
H ₂ SO ₄ 2.5	None	None

^aOne water bottle from each group, replaced weekly, with total plate count and genus of organism determined at the end of each week for six weeks.

^bMean total plate count \pm one standard deviation; n = 6 for each group.

^cGenus of organism isolated

Non-Immunosuppressed Male CD-1 MiceWeight Changes

Prior to being placed on either deionized or acidified drinking water there was no significant difference in body weight between the groups of mice. All groups of animals gained weight over the six-week period of study (see Table 2). Although no significant differences in weight were found until week six, the control animals appeared to gain weight slightly more rapidly than those receiving acidified water. During week six all groups receiving acidified water weighed significantly less ($p < 0.05$) than controls; the overall F value was 3.26, with 4/25 degrees of freedom, $p < 0.05$. This mean weight difference was small and averaged only 9% less for those on acidified water compared to controls during the sixth week. The rate of increase was dependent on pH level as those groups receiving water at pH 2.0 gained approximately .4% of their initial body weights, those groups receiving water at pH 2.5 approximately 40% of their initial body weights, while the controls gained nearly 45% of their initial body weights.

State of Hydration

No significant differences were observed among control and acidified water groups in total body water content. The mean total body water content in ml/100 grams of body weight ranged from 59.2 ± 14.0 for the control group to 65.80 ± 10.4 in those animals receiving water acidified with hydrochloric acid to a pH of 2.0. Animals in the other experimental groups receiving acidified water had intermediate values for total body water content.

TABLE 2
Weight of Non-Immunosuppressed Male Mice

Groups	Time Interval (Weeks)					
	0	1	2	3	4	5
Control	28.56 ± 1.58 ^{ab}	31.75 ± 1.80	33.86 ± 1.97	36.23 ± 2.38	37.80 ± 2.10	39.38 ± 2.38
HCl 2.0	28.68 ± 0.98	29.90 ± 1.57	32.48 ± 1.55	33.55 ± 0.99	34.61 ± 2.22	36.06 ± 2.28
HCl 2.5	26.72 ± 2.02	30.66 ± 1.61	31.75 ± 1.28	34.05 ± 1.05	35.80 ± 1.94	37.08 ± 2.00
H ₂ SO ₄ 2.0	26.93 ± 3.57	29.45 ± 2.00	31.88 ± 2.67	33.26 ± 2.78	34.28 ± 2.70	35.80 ± 2.86
H ₂ SO ₄ 2.5	26.67 ± 2.34	30.05 ± 2.50	31.86 ± 2.27	33.92 ± 2.70	35.72 ± 2.79	37.13 ± 2.75

^a Mean ± one standard deviation; n = 6 for each group

^b Weight in grams

^c Statistically significant difference (p<0.05) when compared to controls

Stomach and Small Intestine pH

The stomach and small intestine pH values are presented in Table 3. Although stomach pH appeared to be somewhat lower in both the glandular and non-glandular portions of the stomach in those animals receiving water acidified to pH 2.0 as compared to control animals, these differences were not statistically significant. No significant differences were found in the pH of any portion of the small intestine examined.

Titratable Stomach Acid Content

No significant differences in titratable stomach acid content was seen between groups. Mean values in milliequivalents/liter for acid in the stomach contents ranged from 1.86 ± 0.72 to 2.37 ± 1.05 .

Disaccharidase Activity of the Small Intestine

The intestinal disaccharidase activities are presented in Table 4. There were no significant differences in sucrase or maltase activity between the control and treatment groups at either pH level using either acidifying agent.

Liver Microsomal Enzyme Activity

No significant differences were found in Cytochrome P450, ethylmorphine-N-demethylase, or aniline hydroxylase activities between mice receiving only deionized water and those mice drinking water acidified to pH 2.0 (Table 5).

Immunosuppressed Male CD-1 Mice

Weight Changes

There were no significant differences in body weights between the five groups of mice studied prior to beginning the acidified water

TABLE 3
Stomach and Small Intestinal pH of Non-Immunosuppressed Male Mice

Groups	Sites			
	Stomach A ^a	Stomach B ^b	Duodenum	Ileum
Controls	2.02 ± 0.58 ^c	4.34 ± 1.76	6.61 ± 0.40	7.61 ± 0.25
HCl 2.0	1.67 ± 0.33	2.47 ± 1.22	6.84 ± 0.06	7.57 ± 0.29
HCl 2.5	2.90 ± 1.28	3.80 ± 2.56	6.76 ± 0.16	7.75 ± 0.22
H ₂ SO ₄ 2.0	1.76 ± 0.62	2.67 ± 1.49	6.79 ± 0.14	7.61 ± 0.21
H ₂ SO ₄ 2.5	2.09 ± 0.76	4.65 ± 1.61	6.76 ± 0.25	7.76 ± 0.50

^a Glandular portion of stomach

^b Non-glandular portion of stomach

^c Mean ± one standard deviation; n = 6 in each group

TABLE 4
Small Intestine Disaccharidase Activity
of Non-Immunosuppressed Male Mice

Groups	Disaccharidase	
	Maltase	Sucrase
Control	59.93 \pm 20.41 ^{ab}	9.30 \pm 5.58
HCl 2.0	67.35 \pm 33.95	8.80 \pm 4.45
HCl 2.5	66.82 \pm 21.25	9.41 \pm 5.09
H ₂ SO ₄ 2.0	72.95 \pm 22.77	10.65 \pm 5.70
H ₂ SO ₄ 2.5	79.75 \pm 25.87	13.50 \pm 7.73

^aMean \pm one standard deviation; n = 6 in each group

^bInternational units of activity/gram of protein

TABLE 5
Liver Microsomal Enzyme Activity
of Non-Immunosuppressed Male Mice

Groups	Enzyme Activity		
	Cytochrome P-450 ^a	Ethylmorphine N-Demethylase ^b	Aniline Hydroxylase ^c
Control	1.13 ± 0.26 ^d	7.23 ± 2.05	1.02 ± 0.12
HCl 2.0	1.19 ± 0.13	7.66 ± 0.63	1.15 ± 0.09

^a μmoles cytochrome P-450/gm microsomal protein

^b μmoles formaldehyde found/gm protein/minute

^c μmoles para-aminophenol/gm protein/minute

^d Mean ± one standard deviation; n = 6, with each n obtained by pooling the livers from three animals.

regimen (Table 6). By the second week of treatment, those animals receiving water acidified to pH 2.0 with sulfuric acid weighed significantly less ($p < 0.05$) than both the immunosuppressed control animals and those animals receiving water acidified with hydrochloric acid to pH 2.5. Beginning with the third week of treatment, those animals receiving water acidified to pH 2.0 with sulfuric acid also weighed significantly less ($p < 0.05$) than non-immunosuppressed control animals. These differences persisted through week six of the study with the exception of the difference seen between the sulfuric acid treated group at pH 2.0 and the immunosuppressed control animals during week six.

The body weight of those animals receiving water acidified to a pH of 2.0 with hydrochloric acid was significantly less ($p < 0.05$) than control and acidified water at pH 2.5 groups during weeks three and four of the study. Although these same differences were present during weeks five and six, they were not statistically significant.

All groups of mice demonstrated substantial increases in weight during the six-week study except the sulfuric acid treated water group at pH 2.0, which showed very little weight gain. All immunosuppressed animals lost weight immediately following exposure to the 450 Rads of gamma radiation. They regained this lost weight in two to three days following exposure and equaled the weights of the non-immunosuppressed controls by the end of the first week of the study. No significant differences in weight gain were apparent between non-immunosuppressed and immunosuppressed control mice, or between either control group and those mice receiving water acidified to pH 2.5 with hydrochloric acid.

TABLE 6
Weight of Immunosuppressed Male Mice

Groups	Time Interval (Weeks)					
	0	1	2	3	4	5
Non-Immunosuppressed						
Control A	27.3 ± 2.3 ^{ab}	29.3 ± 1.7	30.1 ± 1.9	32.2 ± 1.6	34.1 ± 1.5	34.8 ± 2.4
Immunosuppressed						
Control B	28.5 ± 2.8	29.8 ± 1.4	31.2 ± 1.2	31.9 ± 1.2	33.5 ± 1.4	33.9 ± 1.7
HCl 2.0	27.9 ± 1.8	28.3 ± 1.3	29.4 ± 2.1	30.4 ± 1.6 ^c	31.1 ± 0.9 ^{cde}	32.2 ± 1.0
HCl 2.5	29.2 ± 1.1	30.0 ± 2.5	31.2 ± 1.5	33.1 ± 1.0	34.1 ± 2.2	34.9 ± 1.9
H ₂ SO ₄ 2.0	28.2 ± 1.9	28.2 ± 2.1	28.9 ± 1.4 ^{cd}	29.2 ± 1.3 ^{cde}	29.6 ± 0.5 ^{cde}	30.3 ± 1.8 ^{cde}
						31.1 ± 3.0 ^{ce}

^a Mean ± one standard deviation; n = 6 in each group.

^b Body weight in grams

^c Statistically significant difference (p<0.05) when compared to immunosuppressed group receiving HCl treated water at pH 2.5

^d Statistically significant difference (p<0.05) when compared to immunosuppressed controls

^e Statistically significant difference (p<0.05) when compared to controls

State of Hydration

Total body water ranged from 61.20 ± 10.0 for control animals to 59.00 ± 12.8 ml body water/100 gm body weight for acid-treated animals at pH 2.0. No significant differences ($p < 0.05$) were observed between the acid-treated and control groups.

Stomach and Small Intestine pH

No significant differences between groups were found in the pH measurements from any of the areas of the gastrointestinal tract measured with the exception of the measurements from the nonglandular portion of the stomach (Table 7). The mean pH value of the contents of the nonglandular portion of the stomach from those animals receiving water acidified with H_2SO_4 to a pH of 2.0 was significantly less ($p < 0.05$) than the non-immunosuppressed control animals.

Titratable Stomach Acid Content

Mean values for the titration of acid expressed as milliequivalents of acid per liter of stomach contents ranged from a low of 3.76 ± 1.80 to a high of 5.94 ± 3.15 . No significant differences in total titratable stomach acid content were observed between groups.

Disaccharidase Activity of the Small Intestine

No significant differences in sucrase or maltase activity between any of the groups were demonstrated (Table 8).

Liver Microsomal Enzyme Activity

No significant differences between the control and acidified water groups in Cytochrome P450, ethylmorphine-N-demethylase, or aniline hydroxylase activities were found (Table 9).

TABLE 7
Stomach and Small Intestine pH of Male Mice After Six Weeks of Acid Water Consumption

Groups	Sites				
	Stomach ^a	Stomach ^b	Duodenum	Jejunum	Ileum
Non-Immunosuppressed					
Control A	2.25 ± 0.68 ^c	4.63 ± 0.81	6.33 ± 0.41	6.90 ± 0.20	7.51 ± 0.18
Immunosuppressed					
Control B	2.63 ± 1.42	3.52 ± 1.34	6.32 ± 0.42	6.71 ± 0.35	7.49 ± 0.21
HCl 2.0	2.24 ± 0.86	4.22 ± 0.98	6.22 ± 0.16	6.75 ± 0.32	7.70 ± 0.30
HCl 2.5	2.40 ± 0.98	4.17 ± 1.75	6.13 ± 0.15	6.92 ± 0.34	7.54 ± 0.19
H ₂ SO ₄ 2.0	2.11 ± 0.36	2.45 ± 0.88 ^d	6.36 ± 0.25	6.85 ± 0.32	7.54 ± 0.18

^a Glandular portion of stomach

^b Non-glandular portion of stomach

^c Mean ± one standard deviation; n = 6 in each group

^d Statistically significant difference (p<0.05) when compared to non-immunosuppressed controls

TABLE 8
Small Intestine Disaccharidase Activity
of Immunosuppressed Male Mice

Groups	Disaccharidase	
	Maltase	Sucrase
Non-Immunosuppressed		
Control A	25.93 \pm 12.98 ^{ab}	2.94 \pm 1.45
Immunosuppressed		
Control B	24.42 \pm 7.69	2.97 \pm 0.80
HCl 2.0	25.43 \pm 12.05	3.59 \pm 1.22
HCl 2.5	23.16 \pm 12.89	4.46 \pm 0.85
H ₂ SO ₄ 2.0	18.40 \pm 7.29	2.96 \pm 1.24

^aMean \pm one standard deviation; n = 6 in each group

^bUnits of activity/gm of protein

TABLE 9
Liver Microsomal Enzyme Activity
of Immunosuppressed Male Mice

Groups	Cytochrome P-450 ^a	Activity	
		Ethylmorphine ^b	Aniline ^c
Control	1.05 ± 0.14 ^d	6.34 ± 0.54	.655 ± .252
HCl 2.0	1.05 ± 0.18	6.36 ± 0.21	.862 ± .460

^a μmoles cytochrome P-450/gm microsomal protein

^b μmoles formaldehyde formed/protein/minute

^c μmoles para-amino phenol/gm protein/min

^d Mean ± one standard deviation; n = 6, with each n obtained by pooling the livers from three animals.

General Considerations

Food Consumption

No significant differences in food consumption existed between any of the treatment or control groups (Table 10). Daily food consumption ranged from a low of 12.9 ± 0.8 to a high of 15.3 ± 2.4 grams/100 grams of body weight after the first week of the test.

Water Consumption

Male mice consuming water acidified to pH 2.0 drank significantly less ($p < 0.05$) during the fifth and sixth weeks than control mice drinking untreated deionized water (Table 11). In addition, the male mice receiving drinking water at pH 2.0 acidified with either hydrochloric acid or sulfuric acid, drank significantly less ($p < 0.05$) than the male mice drinking water acidified to a pH of 2.5 with hydrochloric acid. No significant differences were found in water consumption between hydrochloric acid and sulfuric acid treated groups at pH 2.0.

Pathology

On gross and histopathologic examination none of the non-immunosuppressed or immunosuppressed animals from any of the treatment or control groups were found to have changes that were considered to be outside of normal limits.

Bacterial Flora from the Terminal Ileum

In general, the organisms isolated from terminal portion of the ileum of the acid treated groups of mice were the same as those organisms isolated from control mice (Table 12). However, it was noted that the use of sulfuric acid at pH 2.0 appeared to lower the incidence of occurrence of some of the organisms, while the use of

TABLE 10
Daily Food Consumption of Male Mice Consuming Acid Water Over a Six-Week Period

Groups	Time Interval (Weeks)					
	1	2	3	4	5	6
Controls	15.7 ± 1.6 ^{ab}	14.9 ± 1.2	14.8 ± 0.4	14.2 ± 0.3	15.3 ± 2.4	15.0 ± 1.3
HCl 2.0	13.6 ± 2.3	14.3 ± 0.4	14.2 ± 0.5	13.2 ± 0.5	13.8 ± 0.4	13.0 ± 0.9
HCl 2.5	14.9 ± 1.9	14.9 ± 1.2	15.0 ± 0.6	14.5 ± 0.6	14.5 ± 1.1	13.3 ± 0.1
H ₂ SO ₄ 2.0	12.9 ± 2.0	14.3 ± 0.4	14.0 ± 0.7	13.6 ± 0.6	14.1 ± 0.1	12.9 ± 0.8
F	0.79 ^c	0.30	1.32	2.49	0.49	1.27

^a Mean ± one standard deviation; n = 2 cages in each group (one immunosuppressed, one non-immunosuppressed)

^b Food consumption in gm/100 gm body weight

^c F values from the one-factor analysis of variance; degrees of freedom are (3/4)

TABLE 11
Daily Water Consumption of Male Mice Consuming Acid Water Over a Six-Week Period

Groups	Time Interval (Weeks)					
	1	2	3	4	5	6
Control	19.1 ± 3.7 ^{ab}	20.2 ± 1.2	19.0 ± 0.7	17.6 ± 1.3	17.7 ± 0.4	16.5 ± 0.7
HCl 2.0	13.6 ± 3.0	13.1 ± 0.8	12.6 ± 0.1	12.1 ± 0.6	11.6 ± 1.3 ^c	11.9 ± 0.8 ^{cd}
HCl 2.5	18.5 ± 7.0	18.2 ± 4.5	17.6 ± 4.4	16.9 ± 4.0	14.7 ± 0.5	15.4 ± 1.3
H ₂ SO ₄ 2.0	12.9 ± 4.0	13.4 ± 1.9	13.1 ± 0.8	12.9 ± 0.2	11.8 ± 1.4 ^c	12.4 ± 0.9 ^{cd}
F	0.95 ^e	2.19	4.14	3.38	11.82	11.10

^a Mean ± one standard deviation; n = 2 in each group (one immunosuppressed, one non-immunosuppressed)

^b Water consumption in ml/100 gm body weight

^c Statistically significant difference (p<0.05) when compared to control groups by Newman-Keuls Testing

^d Statistically significant difference (p<0.05) when compared to HCl 2.5 groups by Newman-Keuls Testing

^e F values from the one-factor analysis of variance; degrees of freedom are (3/4)

TABLE 12
Frequency of Bacterial Isolates from the Terminal
Ileum of Male Mice Following Six Weeks
of Acid Water Consumption

Organisms	Groups				
	Control	HCl 2.0	HCl 2.5	H ₂ SO ₄ 2.0	H ₂ SO ₄ 2.5
Bacillus	14/18 ^a	7/12	7/12	8/12	1/5
Citrobacter	1/18	1/12	1/12	1/12	1/5
E. coli ₁ ^b	6/18	6/12	6/12	2/12	3/5
E. coli ₂	3/18	3/12	3/12	0/12	3/5
Enterobacter	2/18	3/12	1/12	1/12	1/5
Enterococcus	16/18	10/12	7/12	7/12	3/5
Klebsiella	2/18	1/12	2/12	0/12	2/5
Proteus ₁	11/18	6/12	3/12	3/12	0/5
Proteus ₂	4/18	2/12	3/12	0/12	1/5
Staph ₁	2/18	0/12	2/12	1/12	2/5
Staph ₂	3/18	1/12	1/12	2/12	1/5

^a Number of positive isolations/number of animals cultured

^b Subscript numbers indicate separate species or subtype of organisms

hydrochloric acid appeared to have a lesser effect (Table 13). When the number of organisms isolated per animal cultured was analyzed, those mice receiving sulfuric acid treated water at pH 2.0 had significantly fewer ($p < 0.05$) types of organisms. $F = 4.58$ at (4/54) degrees of freedom.

TABLE 13
 Number of Bacterial Species Isolated From
 Each Male Mouse

Groups	N ^a	Number of Isolates/Animal
Control	18	4.22 \pm 1.11 ^{bc}
HCl 2.0	12	3.83 \pm 1.40
HCl 2.5	12	3.41 \pm 1.44
H ₂ SO ₄ 2.0	12	2.25 \pm 1.21 ^d
H ₂ SO ₄ 2.5	5	3.60 \pm 1.14

^aN's obtained by combining the animals from both phases of this study.

^bMean \pm one standard deviation.

^cMean values for the number of species isolated/animal tested within each treatment.

^dStatistically significant difference ($p < 0.05$) when compared to controls.

DISCUSSION

From the results of this study it is evident that consumption of acidified drinking water can have a variety of effects on both immunosuppressed and non-immunosuppressed male mice. While some of these effects were barely discernable, there were other instances in which consumption of acidified drinking water resulted in measurable changes in biological and physiological parameters.

The acidified solutions used in this study remained relatively constant in their hydrogen ion concentration throughout the seven-day periods between water bottle changes. The maximum variation in pH readings with acidified water was approximately 0.07 ± 0.05 , however, with deionized water the maximum variation in pH was 0.31 ± 0.54 . These findings indicate that acidified water maintains a more stable pH with time than deionized water. The stability of the acid concentration in the solutions is in agreement with the findings of McDougall, who reported a similar stability in pH using hydrochloric acid solutions of approximately pH 2.4 (29). These findings, however, differ from the findings of McPherson in which he noted a gradual loss of stability over a seven-day period (30). This difference may stem from the use of tap water in McPherson's study and the use of deionized water in this study.

Even though the pH of the acidified water as well as the deionized water remained relatively constant, the cloudiness noticed in the deionized water, absent from the acidified water solutions, was most likely indicative of changes in the composition of the deionized water. The cloudiness was probably caused by bacterial overgrowth and decomposition of minute amounts of saliva, food particles, and other

materials released into the water while the animals were drinking. The lack of cloudiness in the acidified water was presumably due to inhibition of bacteria and decomposition of these same materials. Culture results suggest that both the numbers and types of bacteria present in the water bottles were substantially less in groups receiving acidified water than deionized water. This agrees with the findings of McPherson, who showed that acidification of water inhibited growth of a variety of bacteria in water bottles for periods of up to seven days (30).

Among those physiological parameters studied in non-immunosuppressed mice, acidified water had a significant effect on weight gain. All groups of mice in this portion of the study started at approximately the same weight; however, those consuming acidified water did not gain weight as rapidly as the control animals consuming only deionized water. This difference became statistically significant by week six with those animals drinking acidified water weighing approximately seven percent less than those drinking deionized water during the sixth week. Since there was no graded response between different pH levels or acidifying agents, it appears that the reduction in weight occurred independently of the acidifying agent. These data suggest that there is probably a threshold for acid concentration of drinking water beyond which weight gain is altered.

The changes in weight gain with acidified water are not entirely consistent with the findings of McPherson, Mullink and Rumke, and Tober-Meyer and Bieniek (30, 31, 42). In all three of these studies the experimental design allowed for a certain degree of accommodation or attenuation of the response to administration of acidified water.

For example, in McPherson's study (30), mice were allowed free access to acidified as well as non-acidified tap water which may have attenuated the effects of the acidified water on weight gain. In the studies by Mullink and Rumke (31), as well as those of Tober-Meyer and Bienick (42), the animals were administered the acidified water for a six- to eight-month period which allowed a substantially longer time for an accommodation of the response to occur and possible masking of small initial differences in weight gain.

The findings of decreased weight gain are in agreement with those studies conducted on acid loading of food (24, 25, 45, 46). In general, these investigators found that consumption of food containing sufficient inorganic acid to lower pH below 2.5 produced a significant reduction in weight. This was true even though the animals studied had access to non-acidified drinking water. Differences in the rate of weight gain could not be attributed to dehydration secondary to decreased water consumption in those groups drinking only acidified water, because total body water was the same in all groups.

Hydrogen ion concentration in the stomach as well as total titratable stomach acid was unaffected by consumption of acidified drinking water in normal animals. This suggests that the consumption of the increased amounts of acid did not exceed the body's ability to compensate by decreasing acid secretion in the stomach. It is certainly possible, however, that consumption of acidified water of lower pH or using other acids may be capable of exceeding the ability of the body to compensate.

In those areas of the small intestine examined, no significant difference in the pH of the contents was found. Failure to find any

differences reduce the possibility that altered intestinal pH resulting from consumption of acidified drinking water could alter intestinal mucosal enzymes in the glycocalyx (9). Indeed, no changes in disaccharidase activity were found. The fact that these enzymes, which have a narrow pH range for optimum activity, are not affected would further suggest that inhibition of nutrient uptake through suppression of enzymatic digestion is not responsible for the changes seen in weight gain in this study.

No significant differences in total cytochrome P450, ethylmorphine-N-demethylase, and aniline hydroxylase activity were found between control animals and those receiving water acidified to pH 2.0. These findings are in agreement with the work of Mullink and Rumke who found no significant difference in the hexobarbital sleeping times of mice treated in a similar fashion (31). This indicates that acid water consumption has no measurable effect on the liver microsomal enzyme system, which is responsible for metabolism of a wide variety of endogenous and exogenous compounds.

The influence of acidified water consumption on weight gain of immunosuppressed male mice showed significant reductions in weight occurring much sooner following the initiation of acid water treatment than in the same study in the non-immunosuppressed mice. This reduction in body weight amounted to a 6 to 10% decrease from control values. These observations indicate that immunosuppression amplifies the effects of acidified water consumption observed in the previous phase of this study. The results further suggest that sulfuric acid causes more pronounced effects at pH 2.0 than hydrochloric acid at this same pH. This observation agrees with the finding of Pritzl in

which he studied the effect of acidification of food with various inorganic acids in chickens (36). He found that sulfuric acid produced significantly lower ($p < 0.05$) weight gain at a higher pH than other inorganic acids. He concluded from this change in weight gain following sulfuric acid administration in the food, that the sulfate ion was responsible for the effects seen. This conclusion is also supported by the observations of Table 6 in this study. These data further suggest that the threshold for acid content of drinking water below which weight gain is affected may be dependent on the anion group present as well as the hydrogen ion concentration.

The results of the state of hydration, hydrogen ion concentration in the stomach and small intestine, stomach acid content, disaccharidase activity of the small intestine, and liver microsomal enzyme activity in immunosuppressed male mice revealed no findings significantly different from the same studies on non-immunosuppressed mice. This suggests that even under stressful conditions such as immunosuppression, consumption of acidified water has no measurable effect on any of these parameters at the levels tested.

While not statistically significant, the mean daily food consumption in those groups receiving water acidified to pH 2.0 was consistently lower than that of groups receiving water acidified to pH 2.5. This observation is compatible with the findings of L'Estrange and Upton as well as others which suggest that a threshold for hydrogen ion intake exists beyond which significant decreased in food consumption can be observed (25). Since mice normally eat relatively small amounts of food even under optimum conditions, it is not surprising that the inherent variability in measuring the small amounts of food consumed

during this study made it difficult to statistically verify differences, and relate decreases in weight gain to decreases in food consumption.

A dramatic effect of acidified water consumption, like the effect on weight gain, was observed in daily water consumption of the male mice. Those mice that received water acidified with either hydrochloric or sulfuric acid to a pH of 2.0 had a daily water intake consistently lower than control mice. Similarly, a decrease in water consumption was noted when the pH 2.0 groups were compared to the pH 2.5 groups, regardless of the acidifying agent used. These differences persisted throughout the entire six-week study period, and were statistically significant during the last two weeks. These findings suggest that water acidified below a pH of 2.5 significantly reduces water consumption and that no degree of accommodation occurs for at least six weeks.

It is interesting to note that when individual treatment groups were examined over the six-week period, their water consumption in grams of water consumed per 100 grams of body weight declined in all groups including controls. This finding suggests that water consumption does not vary linearly with body weight since the body weight of the animals increased over the six-week period. The lack of a linear increase may be related to a decrease in surface area to body mass ratio as the mice gained weight. This change in ratio is related to the degree of insensible water loss and hence will directly affect water consumption (16).

Since no significant differences in water consumption were seen between the two acidifying agents, it is unlikely that the inorganic acid used has any substantial effect on water consumption. In contrast,

the observed difference in water consumption between the pH 2.0 and pH 2.5 groups suggests that the pH of the water is probably an important factor in altering water consumption. This finding is consistent with reports of altered water consumption in animals fed large volumes of acid in their food. In these studies, water consumption increased as food pH was lowered from 3.0 to 2.5 (19, 25). When food was acidified below pH 2.5, water consumption decreased markedly indicating that hydrochloric acid is well tolerated up to a threshold level beyond which measurable effects are observed (45, 46).

The lack of significant gross and histopathologic lesions in any of the groups of immunosuppressed and non-immunosuppressed animals is in agreement with the findings of other investigators which suggest that acidified water consumption produced neither biochemical nor physiologic alterations severe enough to result in detectable histopathologic changes (31).

The examination of bacterial flora of the terminal ileum coupled with the intestinal pH data from both phases of this study suggests that acidified drinking water at the levels examined had no effect on the hydrogen ion content of the small intestine, and that pH therefore was not responsible for the inhibition of the growth of certain kinds of bacteria in the intestine. However, the anion group of the acidifying agent in the drinking water appeared to have a direct inhibiting effect on the numbers of bacterial species isolated. Since this occurred only with the sulfuric acid treated group at pH 2.0 and not at 2.5, the concentration of the anion group seems to be a critical factor and suggests a threshold level for the anion group producing measurable effects.

In general, the acidification of drinking water for laboratory mice is an effective means of reducing the numbers of viable bacterial organisms ingested from the water contained in water bottles. However, in order for this procedure to be effective, water must be acidified beyond a threshold which varies with the acidifying agent. In the case of hydrochloric acid this pH is between 2.0 and 2.5 whereas with sulfuric acid this threshold is greater than 2.5. The data also suggests that inorganic acids other than hydrochloric may be more effective in inhibiting bacterial growth at a higher pH. Therefore, the use of sulfuric acid as an acidifying agent may prevent some of the adverse effects noted in weight gain and water consumption at low pH by permitting higher pH water to be used while maintaining the bacteriocidal efficacy.

It is important to note that the sulfuric acid treated group at pH 2.0, under the additional stress of immunosuppression, did show significant differences in weight gain and pH of the nonglandular stomach when compared to the control and hydrochloric acid treated groups. These findings, coupled with the change in total numbers of bacterial species isolated in the terminal ileum, suggest that the use of the sulfate anion group may tend to magnify small differences in physiologic and metabolic parameters not seen with hydrochloric acid acidification. In the case of the bacterial flora in the terminal ileum the sulfate anion may actually interact with the metabolic processes of certain species thus selecting against their growth. The consequence of such altered intestinal microbial flora is unknown, but may be related to the changes seen in other parameters measured.

The acidification of drinking water with either hydrochloric acid or sulfuric acid is effective in preventing the transmission of bacterial disease through water bottles. However, the changes observed in weight gain, water consumption and bacterial flora of the small intestine in this study point out that the acidification of drinking water may also influence the interpretation of data from animals consuming acidified water. These findings suggest that the acidification of drinking water is not innocuous and that it should be evaluated as an environmental variable whenever it is used. The evaluation of drinking water acidification on a long-term basis is indicated, as well as its potential effects on additional parameters such as development of immunity, reaction to drugs and their metabolism, and the development of neoplasia.

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